Cellular quiescence induced by contact inhibition or serum withdrawal in C3H10T1/2 cells

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Received 14 January 2005; revision accepted 7 March 2005

Abstract. Either confluence or serum withdrawal may cause growth arrest of cultured non-transformed cells. Here, we compared sparsely populated and confluent C3H10T1/2 cells with and without serum-containing medium. The following proliferation-relevant end points were examined: cell-cycle distribution, Ki-67 antigen presence, the level of the von Hippel-Lindau (VHL) protein, and gene expression, determined using a microarray approach. In sparse/logarithmic cultures, the fraction of cells in G₀/G₁ phase increased from 55 to 85% following serum withdrawal. Moreover, the fraction of Ki-67 positive cells dropped from 89 to 47%. In confluent cultures, the majority of cells (80%) were in G₀/G₁ phase and only 25–30% were Ki-67 positive, regardless of serum presence. In both serum-deprived and contact-inhibited cultures, significant and distinct changes in gene expression were observed. Serum deprivation of sparsely cultured cells resulted in significant over-expression of several transcription factors, while confluent cells showed elevated expression of genes coding for *Wnt6*, *uPar*, *Tdag51*, *Egr1*, *Ini1a* and *Mor1*. These results indicate that contact inhibition and serum withdrawal lead to cellular quiescence through distinct genetic and molecular mechanisms.

INTRODUCTION

In many models of non-transformed cells cultured *in vitro*, cell growth arrest occurs either after serum withdrawal or at confluence. The latter effect is descriptively referred to as ‘contact inhibition’ (Abercrombie 1970). Although the pattern of growth arrest is similar in both circumstances, the mechanisms that lead to quiescence seem to differ.

Growth arrest resulting from contact inhibition cannot be abrogated by the addition of fresh media or serum. Proliferation may be restored only by subculturing cells at lower population density. In contrast, cells that ceased to grow by serum withdrawal may re-enter the cell cycle after serum re-supplementation.

It seems that a wide variety of proliferative signals are unable to stimulate the growth of contact inhibited cells, which remain quiescent (Viñals & Pouyssegur 1999). Our previous study

on signal transduction in C3H10T1/2 cells revealed no change in activity of the FAK and ERK1/ERK2 proteins in confluent cells after serum addition (Trembacz *et al.* 2002). Moreover, we found that serum supplementation to low population density cultures, but not to contact-inhibited cultures, resulted in the translocation of protein kinase C to the cell membrane (Miłoszewska *et al.* 1986). Another characteristic change for the densely populated cultures, also described by other researchers, is an elevated level of the von Hippel-Lindau (VHL) protein (Pause *et al.* 1998; Baba *et al.* 2001; Mohan & Burk 2003).

We expected that a further comparative analysis of the two distinct routes to quiescence may reveal some new information pertinent to the still vague mechanisms of contact inhibition. Therefore, the aim of this study was to determine the aspects that distinguish cells stagnated in growth by contact inhibition from cells inhibited by serum withdrawal.

MATERIALS AND METHODS

Cell culture

The C3H10T1/2 cell line (mouse foetal fibroblast) used throughout this study is known for its propensity for growth inhibition at elevated levels of cell-population density (Reznikoff *et al.* 1973). Cells were propagated in Dulbecco's modified Eagle's medium supplemented (unless indicated otherwise) with 10% fetal calf serum (FCS; Invitrogen-Gibco Cell Culture, Carlsbad, CA, USA) and appropriate antibiotics at 37 °C in a humidified atmosphere of 5% CO₂. Sparsely populated (sparse) cultures and confluent cultures here are defined as cultures containing approximately 10 000 and 200 000 cells/cm², respectively. To determine the effects of serum deprivation, cells were plated at 20 000 cells/dish in medium containing 10% FCS. After 24 h, the complete medium was replaced with serum-free medium. After an additional 20 h, serum-deprived cells were harvested and processed as indicated.

Flow cytometry

Cells from either sparse or confluent cultures were harvested by trypsinization, washed once with phosphate-buffered saline (PBS) and re-suspended in ice-cold PBS at a final concentration of 2×10^6 cells/ml. Cell suspensions were processed for cell-cycle distribution and Ki-67 expression using an adaptation of a previously described procedure (Larsen *et al.* 1991). Briefly, 500 µl of permeabilizing DNA staining solution [PBS with Nonidet P-40 (NP40) 0.5% v/v, propidium iodide (PI) 20 µg/ml, RNase 0.25 mg/ml, EDTA 0.5 mM, pH 7.2] were added to 1 ml of cell suspension for 15 min. Next, 40 µl of FITC-conjugated monoclonal Ki-67 antibody or FITC-conjugated control antibody (both from BD Biosciences-Pharmingen, Franklin Lakes, NJ, USA) were added to the cell suspensions and were incubated for 30 min at 0–4 °C with gentle agitation.

Acridine orange DNA–RNA differential staining was performed on ethanol fixed cells as previously described (Darzynkiewicz 1990). All samples were analysed on FACS Vantage flow cytometer (Becton Dickinson Immunocytometry Systems, Franklin Lakes, NJ, USA). DNA profiles were obtained from acridine orange (AO) stained cells. The numerical values of cell-cycle phase fractions were calculated using MacCycle software (Phoenix Flow Systems, San Diego, CA, USA). The G₀ fraction was defined as follows: on the DNA/RNA scattergram (Fig. 1) electronic gate (R3) was set, containing the G₀/G₁ phase cells. The G₀ cells form the left cluster, whereas G₁ (cycling) cells form right cluster. The border between these two clusters was set precisely on the RNA histogram representing only cells in the R3 gate.

Figure 1. An example of flowcytometric measurement of cells stained with AO, a DNA/RNA scattergram; region R3 contains G_0/G_1 cells.

Western blotting

For total protein extraction, cells were scraped and lysed for 30 min on ice in lysing buffer [10 mM Tris, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) with addition of proteases' inhibitors]. The lysates were cleared by centrifugation for 20 min at 10 000 *g* followed by the determination of protein concentration in supernatants using a Bio-RadDC protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

Fifty micrograms of protein/sample were subjected to electrophoresis on 15% polyacrylamide–SDS gels followed by electrotransfer to nitrocellulose membranes (Hybond ECL, Amersham Biosciences, Little Chalfont, UK). Identification of VHL protein was performed using primary rabbit polyclonal VHL antibody and secondary HRP-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and signal detection with ECL Western blotting detection reagents (Amersham Biosciences). Chemiluminescence signals recorded on film were scanned and analysed using Kodak Digital Science 1D Program.

Band intensity measured by densitometry (in arbitrary units) from four independent experiments are presented in Fig. 2 as mean $\pm$ SD.

RNA extraction and gene expression analysis

Total RNA was extracted from C3H10T1/2 cells using TRIzol Reagent (Life Technologies, Rockville, MD, USA) according to the manufacturer's instructions. The RNA was re-suspended in RNase-free water and treated with DNaseI (Atlas™ Pure Total RNA Labeling System, Clontech, Becton Dickinson, Franklin Lakes, NJ, USA). The yield and purity of total RNA were assessed by electrophoresis in denaturing agarose gels and absorbance measurement.

PolyA⁺ RNA was purified using Atlas™ Pure Total RNA Labeling System (Clontech, BD) and was subjected to expression analysis with Atlas™ Mouse 1.2 Array (Clontech, BD). Briefly, 30 μ g of total RNA were mixed with Biotinylated Oligo(dT) and magnetic beads coated with streptavidin. After incubation at room temperature, magnetic beads with bound RNA were washed with appropriate buffers and finally were re-suspended in 3 μ l of RNase-free H₂O. The specific CDS primer mix was added to each sample and then reverse transcription was performed with [α -³²P]dATP (3000 Ci/mmol, 10 μ Ci/ μ l; Amersham Biosciences) and MMLV Reverse Transcriptase (Clontech, BD). The resulting labelled probes were purified from unincorporated

Figure 2. VHL protein content in sparsely populated and in confluent cultures of C3H10T1/2 cells cultured in the presence or absence of serum. 1, C3H10T1/2 cells in the logarithmic phase of growth, cultured in serum-free medium; 2, C3H10T1/2 cells in the logarithmic phase of growth cultured in medium supplemented with serum; 3, confluent C3H10T1/2 cells cultured in serum-free medium; 4, confluent C3H10T1/2 cells cultured in medium supplemented with serum. Whole cell lysates were analysed by western blotting with anti-VHL antibodies. Columns represent bands of intensity measured by densitometry (in arbitrary units) in four independent experiments.

nucleotides and small cDNA fragments with column chromatography and were checked for radioactivity by scintillation counting. The hybridization of cDNA probes to Atlas™ Mouse 1.2 Arrays was performed at 68 °C overnight. After washes, the arrays were exposed to a phosphorimaging screen (Molecular Dynamics, Palo Alto, CA, USA) at room temperature. Analysis of gene expression was performed with Atlas Image 3.2 Software (Clontech, BD).

Arrays were performed on four groups of C3H10T1/2 cells namely: sparse cells propagated in the presence of fetal calf serum (FCS) or without FCS (for 24 h) and confluent cells cultured with FCS or following FCS withdrawal (24 h). A set of 1176 genes was analysed for each group. Comparison of gene expression pattern from contact inhibited and sparsely populated cells following serum withdrawal was performed. A cut of two as minimal level of significance was accepted.

RESULTS

Cell-cycle, growth fraction study

Confluent cultures showed a significantly elevated fraction of cells in the G₀/G₁ phase of the cell cycle compared with sparsely populated cell cultures (81.7% versus 55.6%, respectively). Corresponding differences were seen with the fractions of the S phase cells, which amounted to 17.4% and 41% for confluent and sparsely populated cultures, respectively (Table 1). Changes in cell-cycle distribution were accompanied by a decrease in the Ki-67-positive fraction of cells. In confluent cultures, this fraction dropped to 25% compared with 89% in sparsely populated cultures (Table 1). Sparse cells ceased proliferating after serum withdrawal. The majority of

Table 1. Cell cycle distribution and Ki-67-positive cells in sparsely populated and confluent C3H10T1/2 cultures maintained in complete medium (+FCS) and 20 h after serum withdrawal (–FCS)

	Cell cultures			
	Sub-confluent +FCS	Confluent +FCS	Sub-confluent –FCS	Confluent –FCS
Cell-cycle phase				
G ₀ *	4.7 ± 1.2	6.7 ± 1.4	14.0 ± 2.1	12.6 ± 2.0
G ₀ /G ₁	55.6 ± 1.2	81.7 ± 0.7	85.0 ± 3.7	78.5 ± 0.5
S	41.1 ± 3.0	17.4 ± 1.4	10.8 ± 3.9	18.5 ± 1.1
G ₂ /M	3.3 ± 1.9	0.9 ± 0.8	4.2 ± 1.7	2.9 ± 1.3
Ki-67				
Positive	89.8 ± 0.9	25.5 ± 0.5	47.2 ± 0.3	30.9 ± 3.0

The results are means (± SE) from three independent experiments. Results are expressed as the percentage of total population.

*Percentage of G₀ cells in G₀/G₁ population.

cells became arrested in the G₀/G₁ phase (85%), whereas only 10.4% remained in the S phase. These shifts were accompanied by a significant decrease in the fraction of Ki-67-positive cells observed after serum removal. In contrast, serum withdrawal from confluent cultures had a rather negligible effect on cell-cycle distribution, as well as Ki-67 staining. Slightly elevated levels of cells in the G₀ phase seemed to be related to serum removal rather than to the status of proliferation (Table 1).

VHL study

The level of VHL protein was found to be significantly higher (as tested by Student's *t*-test; $0.05 < P < 0.01$) in confluent rather than in sparse cells. Serum deprivation caused no significant changes in VHL protein level in cells from sparsely populated cultures. However, VHL protein level was significantly decreased in response to serum withdrawal from confluent cultures. The columns on the figure represent mean ± SD of bands intensity measured by densitometry from four experiments (Fig. 2).

cDNA array

Gene array analysis showed that several genes were significantly over-expressed in cells from contact-inhibited cultures compared with sparsely populated cells, following serum deprivation (Table 2). In turn, the serum-deprived cells dramatically differed from their contact-inhibited counterparts by expression of transcription factors. In some cases it was even more than a hundredfold (Table 3). The sparsely populated cultures, when cultured in complete or serum-free media, displayed differences mainly in cell-cycle control and apoptosis-related genes (Table 4).

The rise in transcriptional activity of Wnt6 was found exclusively in confluent contact-inhibited cells as verified by RT-PCR. The results were in agreement with our findings by cDNA methods. These data are not shown here.

DISCUSSION

Non-transformed cells may become quiescent as a result of serum withdrawal or contact inhibition. In our present study we have documented the characteristic features as cell-cycle distribution,

Table 2. Genes over-expressed in contact-inhibited cells as compared with serum-deprived sparsely populated C3H10T1/2 cells

Gene/protein	Ratio conf/log
wingless-related MMTV integration site 6 protein precursor (Wnt6)	35.0
upar1; urokinase plasminogen activator surface receptor (CD87)	31.0
mu-type opioid receptor (Mor-1)	12.7
DNA-repair protein Xrcc1	12.5
mIAP3; inhibitor of apoptosis protein 3	8.1
T-cell death-associated protein (Tdag51)	4.4
G ₁ /S-specific cyclin D1 (Cnd1)	4.1
early growth response protein 1 (Egr1)	4.0
mitogen-activated protein kinase p38 (MAP kinase p38)	3.7
BH3 interacting domain death agonist (BID)	2.8
bone morphogenetic protein 1 precursor (BMP1)	2.8
semaphorin g precursor	2.6
paired mesoderm homeobox protein 1 (PMX1; mHOX)	2.5
structure-specific recognition protein 1 (Ssrp1)	2.4
integrase interactor 1A protein (Ini1a)	2.3
58-kDa inhibitor of RNA-activated protein kinase	2.0

Table 3. Genes over-expressed in serum-deprived sparsely populated cells as compared with contact-inhibited C3H10T1/2 cells (+FCS)

Gene/protein	Ratio log/conf
LIM homeobox protein 2 (Lim2)	140.0
hepatocyte nuclear factor 3 β (Hnf3b)	115.0
<i>Drosophila</i> NK3 transcription factor-related locus 2 (Nkx-3.2; Nkx3b)	81.0
hepatocyte nuclear factor 3 α (Hnf3a)	78.0
gap junction β 3 protein (Gjb3)	75.0
hepatocyte nuclear factor 3 γ (Hnf3g)	70.0
transcription factor 15 (Tcf15)	60.0
paired mesoderm homeobox protein 2A (Pmx2a; Phox2a)	57.0
endothelial pas domain protein 1 (Epas1)	57.0
gap junction α 5 protein (GJA5)	55.0
brachyury protein (T protein) – T-box gene	54.7
vimentin (Vim)	54.5
transcription factor Fkh-5	54.0
t-box protein 13 (Tbx13)	52.0
sine oculis-related homeobox protein 4 homolog (Six4);	52.0
eyes absent homolog 1 (Eya1)	52.0
paired box protein 2 (Pax2)	49.0
aryl hydrocarbon receptor nuclear translocator 2 (Arnt2)	48.0
eyes absent homolog 3 (Eya3)	45.5
cut-related homeobox cux-1 (Cutl1)	44.0

Only the 20 genes with the most elevated expression are presented.

Table 4. Differences in the expression of genes coding for cell-cycle and apoptosis-related proteins in sparsely populated C3H10T1/2 cultures in complete or in serum-free medium

Gene/protein	Ratio log FCS/ log-FCS
ran GTPase activating protein 1 (Rangap1)	16.0
p55cdc	14.0
interleukin 1 receptor antagonist	6.5
bcl-2 homologous antagonist/killer (Bak1)	5.3
G ₂ /M-specific cyclin B2 (Ccnb2)	5.2
mIAP3; inhibitor of apoptosis protein 3	5.1
G ₁ /S-specific cyclin D1 (Ccnd1)	4.6
mitogen-activated protein kinase p38 (MAP kinase p38)	3.7
survival of motor neurone (hSMN)	3.3
BH3 interacting domain death agonist (BID) (BCL-family)	2.1
rac α serine/threonine kinase (RAC-PK- α)	0.5
nucleoside diphosphate kinase B	0.5
programmed cell death 2	0.5
bAX membrane isoform α	0.5

Ki-67 antigen expression and gene expression, in cells under both these conditions. Approximately 80% of confluent cells were found to be in the G₀/G₁ phase, independent from serum presence. In addition, 75% of contact inhibited cells were already negative for Ki-67, and serum withdrawal did not affect that fraction. Moreover, contact-inhibited cells similarly accumulated in G₀/G₁, either in the presence or in the absence of serum. This pattern resembles the results for 3T3 cells described by Endl *et al.* (2001), who showed that a significant decrease in the percentage of Ki-67-positive cells parallels the emergence of contact inhibition. The G₀ fraction, as determined by the flow cytometry method, surprisingly showed a slightly higher percentage of G₀ cells in serum-depleted cultures independent of their densities as compared with cells cultured in the presence of serum.

Although no essential differences were found in proliferative parameters of quiescent sparse and confluent cells, altered gene expression was observed. The biggest differences were found in activity of the gene coding for Wnt6 protein, the ligand in the Wnt pathway involved in developmental processes such as crest formation. The Wnt pathway plays a very important role in tissue regeneration and stem-cell renewal, in particular with regard to regulation of asymmetric cell division (Beachy *et al.* 2004; Liu *et al.* 2004). The same pathway was found to be oncogenic when deregulated in mammalian tissue (Taipale & Beachy 2001). The elevated Wnt6 level observed in confluent contact inhibited cells might result from regenerative proliferation, especially that 17% of these cells were in the S phase at the time (Table 1). An additional argument to support this hypothesis is that the Wnt6 transcript was not expressed in either subconfluent or confluent cells cultured in serum-free medium, as documented by cDNA arrays and RT-PCR. Accepting this point of view, contact inhibition can be considered as a balanced mode of regeneration accompanied by an elevated level of Wnt6. Thus, the rise of Wnt6 expression can be considered to be a consequence of the phenomenon of contact inhibition. Why confluent cells begin production Wnt of requires further experimental work.

Mor1 and *uPar* were two further genes whose elevated expression was restricted to confluent cells cultured in medium containing FCS. *Mor1* encodes a mu-type opioid receptor. The two major functions of this mu opioid receptor are known to involve the modulation of intracellular

cAMP and that of specific ion channels (Yu *et al.* 1996). The expression of the *Mor1* gene is regulated by a cAMP-dependent pathway (Lee & Lee 2003). In turn, cAMP is regarded as a negative regulator of cell proliferation and cell-cycle traverse (Bertram 1979, 1982; Janik & Miłoszewska 2002). The elevated expression of *uPar* in contact-inhibited cells is rather uncommon as the receptor for a plasminogen activator is a component of the uPa system. This system plays an important role in cell migration and therefore in tumour invasion and angiogenesis (Los *et al.* 1999).

The two next genes in which expression was enhanced in confluent contact-inhibited cells, regardless of serum presence, were those coding for the early growth response protein (*Egr1*) and integrase interactor 1A protein (*Ini1a*). Interestingly, the forced expression of *Ini1a* in malignant cells that normally do not express it (e.g. rhabdoid tumour cell lines) induced accumulation of cells in G₀/G₁ (Betz *et al.* 2002). Moreover, *Ini1a* overexpression has been reported to repress transcription of cyclin D1 via histone deacetylation at the promoter region of the *Ccnd1* gene (Zhang *et al.* 2002). In our model, however, cDNA array analysis revealed no changes in *Ccnd1* expression. *Egr1*, however, encodes a protein involved in cellular response to mitogens, growth factors or stress stimuli (Nair *et al.* 1997). Recently, Calogero *et al.* (2004) have suggested that *Egr1* might act as a tumour suppressor gene, because its reintroduction into malignant cell lines lacking its expression reduced their growth in culture.

The expression level of the gene coding for cyclin D1 is slightly higher in contact-inhibited cells cultured in full medium, than in quiescent cells in serum-free medium. This may reflect that cells in serum-free media, regardless of their population density, displayed higher percentage of G₀ cells.

The deprivation of growth factors that are normally provided by serum might also induce non-transformed cells to exit the cell cycle and consequently initiate quiescence in them. C3H10T1/2 cells cultured at low population density exhibit lower proliferation capacity in medium lacking FCS. Their growth arrest is indicated by accumulation of G₀/G₁ cells and the lower percentage of Ki-67 positive cells, as compared with cells cultured in complete medium. Ki-67 is well established as a marker of cell proliferation and is expected to disappear after cell transit to a non-proliferative state, such as G₀. However, the term 'non-proliferative state' is often only vaguely defined (Scholzen & Gerdes 2000). In our experiments, about 14% of serum-deprived cells in low population density cultures could be accounted as being in G₀.

The 'sparse' cells, after serum withdrawal, displayed dramatic elevation in expression of genes coding for transcription factors (TFs). It is well established that VHL degrades HIF (hypoxia inducible factor) – one of the key regulators of cell physiology. However, the elevated level of expression of transcription factors here cannot be fully explained by a lower content of VHL protein in sparse density cells after serum withdrawal. High proliferation capacity of sparse C3H10T1/2 cells cultured in medium supplemented with FCS is reflected by the pattern of gene expression in these cells. We have observed a high expression of some genes encoding proteins involved in cell-cycle progression such as *Rangap1*, *p55cdc*, *Ccnb2* and *Ccnd1*. In contrast, the gene coding for an anti-proliferative factor *Tob* was over-expressed in serum-deprived cells.

Serum withdrawal from non-transformed fibroblasts has also been found to promote apoptosis (Kulkarni & McCulloch 1994). A further study has indicated that both transformed and normal cells exit the cell cycle during prolonged serum deprivation (Darzynkiewicz *et al.* 2003), and the protection of serum-deprived cells from apoptosis was suggested to involve the VHL protein. Specifically, Pause *et al.* (1998) showed that renal cell carcinoma 786-O cells may exit cell-cycle yet survive serum deprivation if they are transfected with wild-type VHL. Surprisingly, we have found the level of VHL protein to be significantly decreased in serum deprived contact-inhibited cells. The possible explanation for this is that cell-to-cell contacts are disrupted

during starvation, somewhat analogous to the effect of treatment with EDTA, which leads to VHL decrease (Mohan & Burk 2003).

The reported characteristics of contact-inhibited and serum-deprived cells seem relevant to a broader context of how tumour cells gain a growth advantage. Tumour cells appear to develop special mechanisms that allow them to continue proliferation, even at high population densities, or to avoid apoptosis under conditions of depleted supply of growth factors. Data obtained in the model of C3H10T1/2 cells has documented the existence of distinct links between different culture conditions, cell-cycle distribution and the expression of proliferation markers, as well as to changes in VHL level. Collectively, the findings are consistent with the possibility that suppression of proliferation after serum withdrawal might be related to cells' slight accumulation in G₀, whereas contact inhibition might lead to cell-cycle arrest in the G₁ phase. Moreover, our results point to several specific genes, most importantly *Wnt6*, *uPar*, *Mor1*, *Egr1* and *Inila*, whose expression might be tightly related, perhaps controlled, by contact inhibition. Further studies are warranted to clarify the roles of these and other proteins in whom expression was modified by contact inhibition or growth factor deprivation.

ACKNOWLEDGEMENTS

This study has been supported by a Cancer Center intramural grant.

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